Avidity Effects of MinE07, an Anti-EGFR Aptamer, on Binding to A431 Cells

Presented by: Viswatej Avutu

In partial fulfillment of the requirements for graduation with the
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TABLE OF CONTENTS

ABSTRACT

Cell growth, differentiation, and proliferation are all carefully regulated processes. Disruptions in these processes are often associated with malignant tumors. The epidermal growth factor receptor (EFGR), part of the ErbB family of receptors, is known to play a pivotal role in regulating numerous cell growth processes including morphology, differentiation, proliferation, and apoptosis in certain cell types. Overexpression or elevated levels of EGFR activity is associated with many different types of cancers. Numerous targeted anti-EGFR therapies have been developed, including monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors. Apatmers provide an attractive alternative to monoclonal antibodies due to their ease of synthesis and lack of immunogenicity.

J18 and E07 are two aptamers which were selected for against EGFR. Due to 2'-fluoro pyrimidines modification, E07 was chosen for *in vivo* applications. E07 was further remodeled to a minimal length construct that still retained binding affinity for EGFR. A cell growth assay using E07 and the anti-EGFR mAb, Cetuximab, revealed that a much larger dose of aptamer was needed to achieve the same level of growth inhibition as Cetuximab. In an effort to improve the efficiency of E07, an experiment was designed to improve the K_d of E07 with avidity effects – supradditive effects observed upon dimerization or multimerization of monomers. Having been observed with peptides, it was hypothesized that nucleic acids might also display such avidity effects.

Five dimeric constructs of the minimized E07 (MinE07) aptamer were tested using flow cytometry assays on A431 cells. Two variables were also tested: the orientation of the monomers in the dimeric construct and the distance separating the two monomers. Constructs were assembled in three different schemes. First, DNA organizers containing fluorophores (fluorescein) were used to direct formation. In a second strategy, the extensions added to

MinE07 directed the formation of the dimer. Lastly, the dimer was created via transcription off of an ssDNA template.

FACS data revealed that none of the constructs significantly produced avidity effects. However, Construct 3 did inconsistently demonstrate slight avidity effects. Depending on the conditions of the A431 cells, the cell surface and subsequent assays can change dramatically. The head-to-tail orientation proved to be more promising in permitting avidity effects. Because no significant avidity effects were seen, the effect of intra-aptamer distance on binding affinity could not truly be studied.

INTRODUCTION

The epidermal growth factor receptor (EGFR), a member of the ErbB/HER family of Type I transmembrane tyrosine kinase receptors, has been implicated in numerous cancers, including breast, lung, and colorectal, and head and neck squamous cell cancers.[1-5] In normal physiology, ErbB receptors play central roles in the development and growth of tissues by moderating cell differentiation and morphology.[2] Various growth factors, including the epidermal growth factor, serve as ligands to this family of receptors. Upon binding of the ligand, homo- or heterodimerization of receptors occurs, leading to autohphosphorylation of tyrosine residues on the cytoplasmic domains.[4] Two primary downstream signaling pathways are then initiated: the Ras signaling cascade and the PI3K signaling cascade.[4] These downstream cascades lead to cell division and subsequent differentiation and proliferation. In addition, dimerized EGFR can also be internalized into early endosomes. From these early endosomes, EGFR can either be recycled back to the membrane or degraded after processing.[6]

Overexpression of ErbB receptors, especially of EGFR, has been shown to cause erroneous and uncontrolled cell proliferation, leading to malignant tumors.[1] In addition, increased activation of EGFR has been linked to decreased apoptosis, increased metastasis, and increased angiogenesis, all hallmarks of cancerous cells.[2] Thus, anti-EGFR therapy is a major area of research for anti-cancer therapeutics. Current anti-EGFR agents include monoclonal antibodies against EGFR (Cetuximab) and tyrosine kinase inhibitors (Geftinib).[2-3,5] However, there are some innate problems with using monoclonal antibodies as drugs: use of a biological system, difficulty with chemical modifications, and significant immunogenicity.[7] This has complicated their use as chemotherapeutics. Similarly, the clinical effectiveness of tyrosine kinase inhibitors is limited due to resistance development in cancer cells to these inhibitors as well as a general lack of response by tumors in the general population across various types of cancers.[8]

Aptamers are single-stranded oligonucleotides, either of deoxyribonucleic acid or ribonucleic acid composition, generated from a combinatorial pool and a stringent selection process, such as SELEX, Systematic Evolution of Ligands by Exponential Enrichment, with very high affinity towards a specific target.[9] This high affinity arises from the unique three-dimensional folding of the aptamer, allowing it to interact specifically with its target. Binding affinities generally range in the nanomolar to picomolar range, comparable to those of monoclonal antibodies.[8] Due to their nucleic acid origin, aptamers are more easily synthesized by chemical means or can also be enzymatically amplified.[10] Furthermore, various chemical modifications can be made that give aptamers distinctive properties: labeling with fluorescent or radioactive probes for imaging purposes, conjugations to various compounds (siRNA and peptides), and increased stability for *in vivo* applications.[11-13] With many of the advantageous properties of monoclonal antibodies but few of their disadvantages, aptamers are a relatively new class of therapeutic agents and a potentially promising class of clinical drugs.

J18, an aptamer selected against EGFR by Li *et al*., was characterized by a K_d of about 7 nM. A similar selection with 2'fluoro- pyrimidine modified ribonucleotides was performed, resulting in E07, in order to extend aptamer half-life for *in vivo* applications.[14] In order to prevent excessive aptamer length upon addition of extensions and chemical modifications, minimization was used to produce a construct with the minimal length of aptamer that still retained function: MinE07.[15] A431 cells, a model epidermoid carcinoma cell line, are known to overexpress EGFR and thus, serve as the model cell line for studying EGFR and the efficacy of anti-EGFR therapies.[16] Because EGFR is internalized upon ligand-binding and dimerzation, ligands such as anti-EGFR aptamers are also internalized.[6] Internalization of aptamers allows for possible targeted drug delivery and imaging potential. It has been shown that J18 binds to and internalizes into A431 cells by flow cytometry.[17]

Comparison of E07 and other anti-EGFR therapeutics reveals that despite E07's high affinity to EGFR, its relatively large K_d prevents it from being clinically useful (unpublished data). Thus, in order to increase its efficacy, various dimeric MinE07 constructs were made with the idea that through avidity effects, dimerized MinE07 would bind at a higher rate.[18] Multivalency effects have been shown with polypeptides and antibodies to increase efficacy.[19] Avidity effects have also been observed in receptor signaling.[20-21] It was thus hypothesized that aptamers may also display avidity effects upon multimerization due to the similar nature of action of antibodies and aptamers. To test whether the dimeric MinE07 constructs have better binding rates, flow cytometry was used to assess fluorescence (fluorophores were bound to constructs).

METHODS AND MATERIALS

Synthesis of Extended MinE07 Aptamers

MinE07 sequence (minimization data not published) used was: 5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCC-3'. 3' hybridization extensions and a 5' probe extension to the minimized aptamer were made with the use of various primers (**Table 1**). All primers and DNA organizers containing fluorescein (**Table 2**) were ordered from IDT (Integrated DNA Technologies, Coralville, IA). MinE07 ssDNA template was polymerase chain reaction-amplified (Invitrogen, Carlsbad, CA) to make dsDNA. dsDNA constructs were then transcribed using a Durascribe® T7 Transcription Kit with 2'-fluoro pyrimidines (Epicentre, Madison, WI) to make modified RNA aptamer constructs. RNA constructs were then gel purified using an 8% denaturing polyacrylamide gel, eluted, and precipitated. Sequences are listed in **Table 3**. A dimer version of MinE07, containing the full-length E07 aptamer, 5 As, and the minimized E07 aptamer, was also ordered from IDT: 5'-GGACGGTTCCGGCTTTGACATGCTTTTCTACGGCGATTAAATCCGTCCTTTTTGGACGGTTCCGGCTTTGACATGCTTTTCTACGGCGATTAAATCCGTCCTATAGTGAGTCGTATTATC-3'. ssDNA template of this dimer construct was PCR-amplified and transcribed as described above

to produce the MinE07.Dimer (see **Table 3**). All RNA constructs and DNA organizers were stored in nuclease-free water (Epicentre) at -20°C until use. MFold was used to verify that extensions caused no significant changes in the secondary structure of the minimized aptamer.[22,23]

Primer Name	Primer Sequence
Forward	5'-**GATAATACGACTCACTATA**GGACGGATTTAATCGCCG-3'
Forward.Probe	5'-**GATAATACGACTCACTATA**GGATCCGGAATCTCCGATCTGGACGGATTTAATCGCCG-3'
Forward.Mutant	5'- **GATAATACGACTCACTATA**GGAGGTTAGACAGCAGGC-3'
Reverse.A	5'-CATTTAGGACCAACACAAGGACGGTTCCGGCTTTGA-3'
Reverse.B	5'-CATCACCACTTCTACTTAGGACGGTTCCGGCTTTGA-3'
Reverse.C	5'-TAAGTAGAAGTGGTGATGGGACGGTTCCGGCTTTGA-3'
Reverse.MutantA	5'-CATTTAGGACCAACACAAACAGTTGATTGTTCTGTG-3'
Reverse.MutantB	5'-CATCACCACTTCTACTTAACAGTTGATTGTTCTGTG-3'

Table 1: All primers were ordered from IDT. Bolded sequences indicate the T7 polymerase promoter and underlined sequences indicate hybridization extensions.

DNA Organizer Name	DNA Organizer Sequence
Spacer 3	5'-/56-FAM/CATTTAGGACCAACACAA/iSpC3/CATCACCACTTCTACTTA-3'
Spacer 9	5'-/56-FAM/CATTTAGGACCAACACAA/iSp9/CATCACCACTTCTACTTA-3'
Spacer 18	5'-/56-FAM/CATTTAGGACCAACACAA/iSp18/CATCACCACTTCTACTTA-3'
Probe	5'-AGATCGGAGATTCCGGATCC/36-FAM/-3'
Linker.A	5'-AACACAACCAGGATTTACAAAGCTCTCAGCTCACAGAAC/36-FAM/-3'
Linker.B.5	5'-ATTCATCTTCACCACTACAAAAAAAAGTTCTGTGAGCTGAGAGC-3'
Linker.B.10	5'-ATTCATCTTCACCACTACAAAAAAAAAAAAAGTTCTGTGAGCTGAGAGC-3'
Linker.B.15	5'-ATTCATCTTCACCACTACAAAAAAAAAAAAAAAAAAGTTCTGTGAGCTGAGAGC-3'

Table 2: All DNA organizers were ordered from IDT. The fluorophore used was fluorescein.

Construct Name	Construct Sequence
MinE07.A	5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCCUUGUGUUGGUCCUAAAUG-3'
MinE07.B	5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCCUAAGUAGAAGUGGUGAUG-3'
MinE07.C	5'-*GGAUCCGGAAUCUCCGAUCU*GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCC CAUCACCACUUCUACUUA-3'
MinE07.MutantA	5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCCUUGUGUUGGUCCUAAAUG-3'
MinE07.MutantB	5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCCUAAGUAGAAGUGGUGAUG-3'
MinE07.Dimer	5'-GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCCUAAGUAGAAGUGGUGAUG**AAAAA**GGACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCGGAACCGUCC-3'

Table 3: Sequences of aptamer constructs used. Underlined sequences indicate hybridization arms and italicized sequence indicates probe hybridization arm. The bolded sequence is the additional As added between the full-length E07 and the minimized E07.

Synthesis of SMCC DNA Organizer

Two constructs, 5'-/56-FAM/CATTTAGGACCAACACAA/3ThioMC3-D/-3' and 5'-CATCACCACTTCTACTTAAAAAAAAAAA/3AmM/-3' were ordered from IDT. Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Thermo Scientific, Rockford, IL) was used to crosslink these two constructs to form a fluorescein-DNA organizer. Sulfo-SMCC was first resuspended in dimethylformamide (Sigma-Aldrich, St. Louis, MO) to a final concentration of 20 µg/µL. 20 µL of 500 µM amine-containing DNA was then reacted with 5 µL sulfo-SMCC in PBS to a final concentration of 1X PBS 10% DMF (v/v) at room temperature overnight. The malemide-activated DNA was subsequently purified using a G-25 Column (Roche, Indianapolis, IN). To reduce the disfulfide bonds, 20 µL of 500 µM thiol-containing DNA was reacted with 5 µL of 0.5M TCEP (Thermo Scientific) in PBS and DMF to a final concentration of 1X PBS 10% DMV (v/v) at room temperature overnight. The reduced sulfhydryl-containing DNA was similarly purified using a G-25 column. Both reactive DNAs were then incubated together at room temperature overnight. After the reaction went to completion, the SMCC DNA organizer was gel purified using an 8% denaturing polyacrylamide gel, eluted,

and precipitated. The final sequence was: 5'-56-FAM/CATTTAGGACCAACACAA-/HS/-SMCC-/$_2$HN/-AAAAAAAAAAATTCATCTTCACCACTAC-3'. It was stored in nuclease-free water at -20°C until use.

Formation of Dimer MinE07 Constructs

Dimer constructs were formed from annealing the extended MinE07 apatmers to either DNA organizers or to themselves. Construct 1 was formed from annealing MinE07.A and MinE07.B with Spacer 3, 9, or 18. Construct 2 was formed from hybridizing the 3' extensions of MinE07.B and MinE07.C and annealing Probe to the 5' extension of MinE07.C. Construct 3 was formed from annealing MinE07.A and MinE07.B to the SMCC DNA organizer. Construct 4 was formed from annealing MinE07.A and MinE07.B to Linker.A and LinkerB.5, B.10, or B.15 (Linker.A contained the fluorophore). Construct 5 was the transcribed MinE07.Dimer.

Native Gel Electrophoresis

Native gel electrophoresis was used to verify the formation of MinE07 dimers. Dimer constructs were prepared by mixing equal amounts of extended aptamers with the fluorophore-containing DNA organizers. Each solution was then heated to 70°C for 3 minutes and cooled to 25°C at a rate of 1°C/sec. 1 µL of 50% glycerol was added to 10 µL of 40 µM dimer constructs and then loaded onto an 8% native (non-denaturing) polyacrylamide gel. After gel electrophoresis, the gel was stained with SYBRGold (Invitrogen) prior to imaging on a PhosphorImager.

Cell Culturing

The A431 cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA). The cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; ATCC) with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma-Aldrich).

Flow Cytometry

Media from A431 cells was first removed, followed by a one-time wash with 10% FBS Dulbecco's Phosphate Buffered Saline (Invitrogen). 1 mL of 0.25% trypsin-EDTA (Invitrogen) was then added to trypsinize the cells. After approximately 5 minutes (or upon visual confirmation of cell detachment) at 37°C and 5% CO_2, the reaction was terminated by the addition of 4 mL of 10% FBS DMEM. Approximately 5 x 10^6 cells, as counted with a hemocytometer, were used for each flow cytometry assay. The cells were pelleted and washed three times with 100 µL of binding buffer (1X DPBS with 5 mM $MgCl_2$) and resuspended into 100 µL binding buffer aliquots, one for each reaction in the assay: A431 cells only, each aptamer with the fluorophore-containing DNA organizer, and the dimeric construct. Aptamer was first prepared by annealing each monomer or dimer to the DNA organizer at 70°C for 3 minutes and cooling to 25°C at a rate of 1°C/sec. Monomeric or dimeric aptamer constructs were added to a final concentration of 100 nM in each 100 µL reaction aliquot. A431 cells were incubated with aptamer constructs for 30 minutes. After incubation, cells were once again washed three times with 100 µL of binding buffer and resuspended in 300 µL of binding buffer. Fluorescence was assayed using a FACSCalibur (Becton Dickinson, San Jose, CA). 10,000 events were collected for each reaction using BD CellQuest Pro Software. Analysis made use of the FLH-1 detector at a voltage of 412 mV without gating. A plot of FSC-H vs. SSC-H was used to verify viability of cells.

Competition assays were performed using FACSAria (Becton Dickison) and a FITC-H detector. All other conditions were the same. MinE07.A was used to establish the initial baseline that MinE07 and MinE07.Dimer competed against. 1:1 and 1:4 of MinE07:competitor ratios were used.

RESULTS AND DISCUSSION

Verification of Dimer MinE07 Constructs

The native gel (**Figure 1**) shows the formation of the dimer constructs 1, 2, and 3. Construct 5 was verified with the use of a 8% denaturing gel (data not shown). Construct 4 showed similar dimer formation as Construct 1 (data not shown).

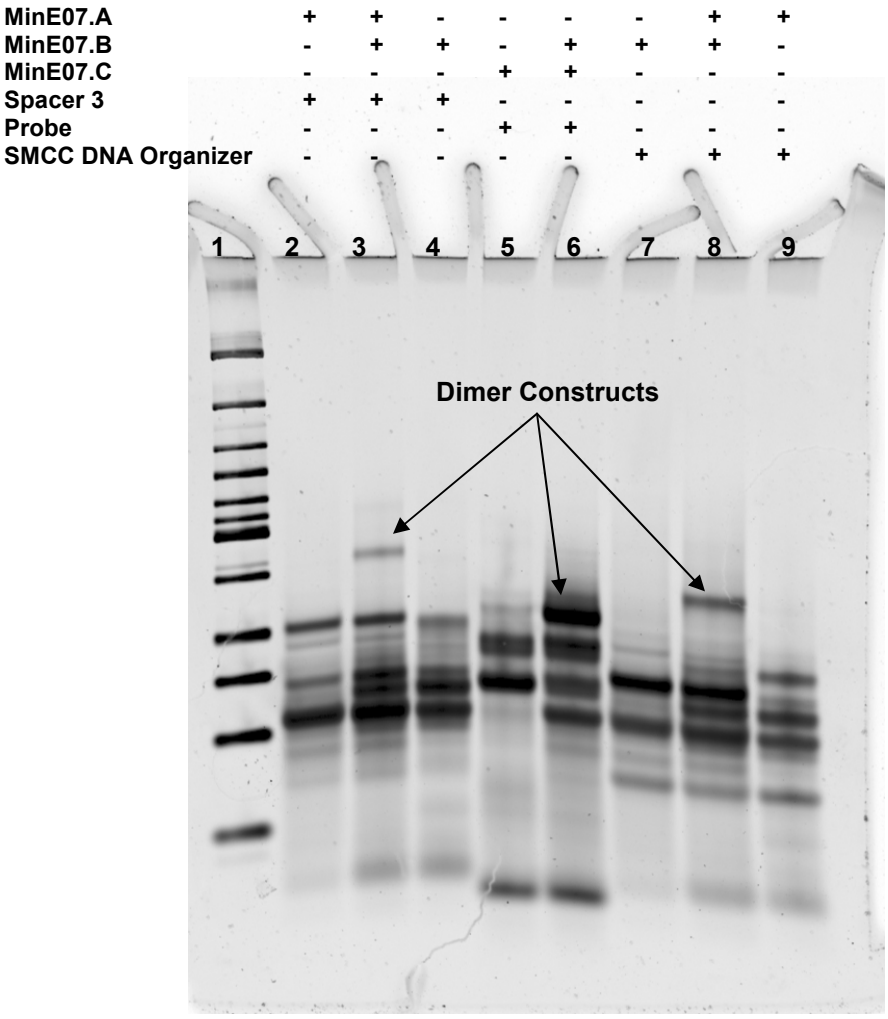

Figure 1: 8% native gel. Lanes contain: **1** – 25 bp Ladder (Invitrogen); **2** – MinE07.A annealed to Spacer 3; **3** – MinE07.A + MinE07.B annealed to Spacer 3 (Construct 1); **4** – MinE07.B annealed to Spacer 3; **5** – MinE07.C annealed to Probe; **6** – MinE07.B + MinE07.C annealed to Probe (Construct 2); **7** – MinE07.B annealed to SMCC DNA organizer; **8** - MinE07.A + MinE07.B annealed to SMCC DNA organizer (Construct 3); **9** – MinE07.A annealed to SMCC DNA organizer.

It is evident that sufficient formation of Constructs 2 and 3 occurs such that enough dimeric aptamer constructs would be available during flow cytometry assays to see possible avidity effects. Construct 1 and 4, however, may not form enough dimerized construct to significantly impact binding and show avidity effects.

Flow Cytometry Analysis: Construct 1

All three Spacers (3, 9, and 18) were tested to see if affecting the distance between the aptamers would have an effect on binding. FACS data (**Figure 2**) indicates that, regardless of the length of the internal spacer, the dimer construct does not show avidity. However, based on native gel analysis (**Figure 1**), avidity may not be evident because only a small percentage of the aptamer population incubated with A431 cells formed dimers. In addition, the orientation of the aptamers may not allow for binding two EGFR dimers in the correct orientation. MinE07.A and MinE07.B are attached in a head-to-head orientation (with their 5' ends facing the same direction). To test whether a head-to-tail orientation, one in which the 5' ends face in opposite directions, subsequent apatmers were made in the head-to-tail orientation. It should also be noted that MinE07.B binds stronger to EGFR than MinE07.A as indicated by the greater rightward shift in fluorescence. This could be due to additional affinity provided by the 3' extension sequence.

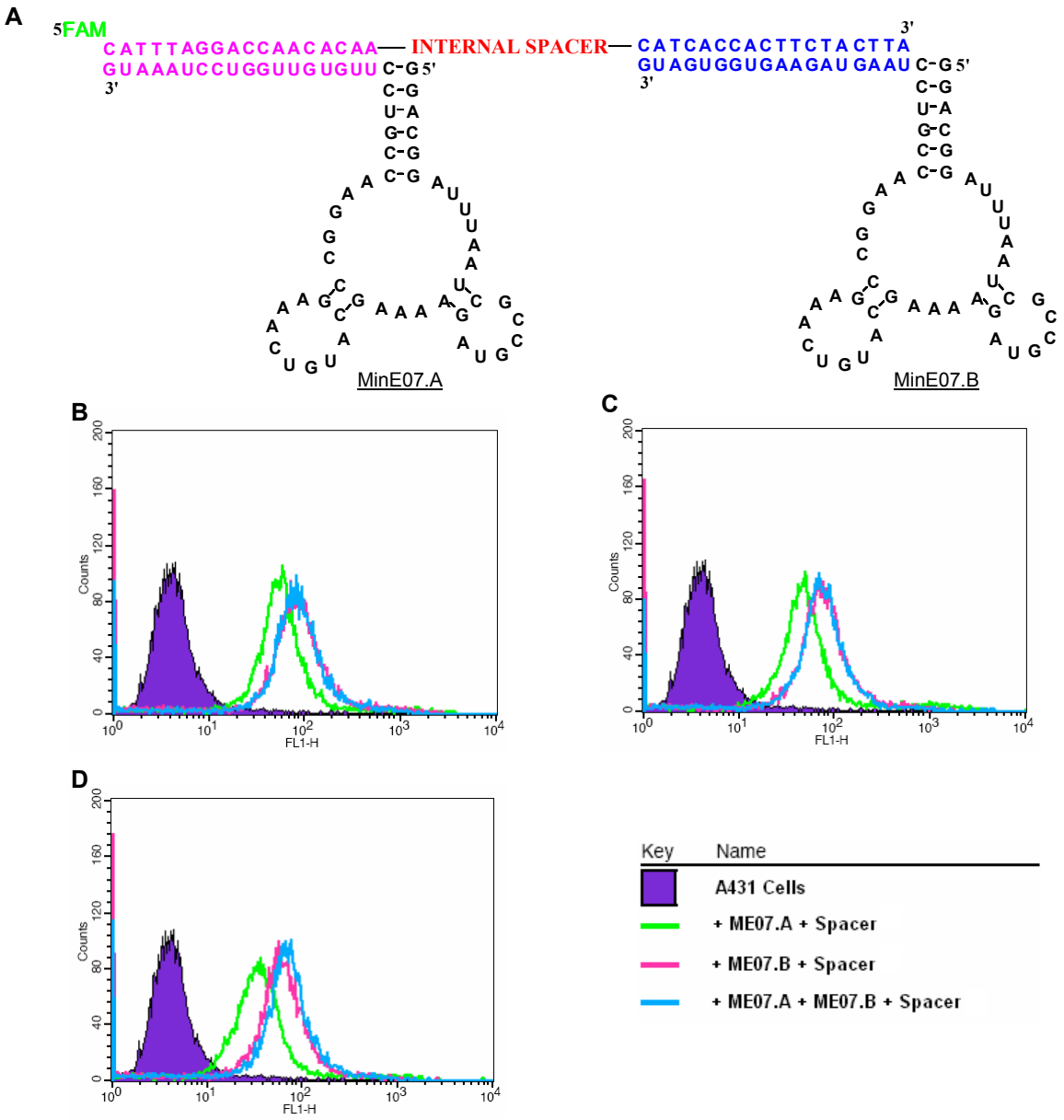

Figure 2: Fluorescein-bound aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.A annealed to DNA organizer Spacer (green line), MinE07.B annealed to Spacer (pink line), and MinE07.A and MinE07.B annealed to Spacer (blue line). (**A**): Pink sequences represent the hybridization between MinE07.A and its complement in Spacer; blue sequences indicate the hybridization between MinE07.B and its complement in Spacer. Internal spacers (IDT) were 3 carbon, 9 carbon, or 18 carbon glycol spacers. (**B**): FACS data of Construct 1 using Spacer 3. (**C**): FACS data of Construct 1 using Spacer 9. (**D**): FACS data of Construct 1 using Spacer 18.

A composite of Construct 1 with different internal spacer lengths (**Figrue 3**) shows no differences in binding. This implies that EGFR monomers may be spaced farther apart than the

flexibility afforded by the DNA organizer Spacer or that Construct 1 did not induce EGFR dimerization.

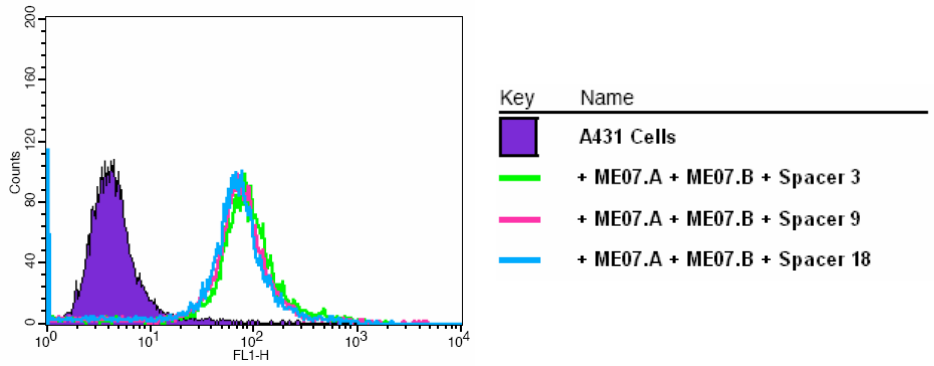

Figure 3: FACS data of Construct 1, comparing the different internal spacers which differed by length. ME07 refers to MinE07.

Flow Cytometry Analysis: Construct 2

Construct 2 was made from hybridizing the 3' extensions of MinE07.B and MinE07.C together such that they dimerized in a head-to-tail orientation (with their 5' extensions facing opposite directions). The fluorphore-containing compound was Probe that hybridized to the 5' extension of MinE07.C. FACS data (**Figure 4**) reveals that the affinity of MinE07.C for EGFR was greatly decreased as indicated by a nearly baseline fluorescence signal. Annealing MinE07.B to MinE07.C and Probe did not rescue binding affinity despite a change in the orientation from head-to-head to head-to-tail. The three-dimensional structure required for MinE07 to correctly bind to EGFR was most likely destroyed with the 5' and 3' extensions made to MinE07.C and the annealing of Probe and another aptamer.

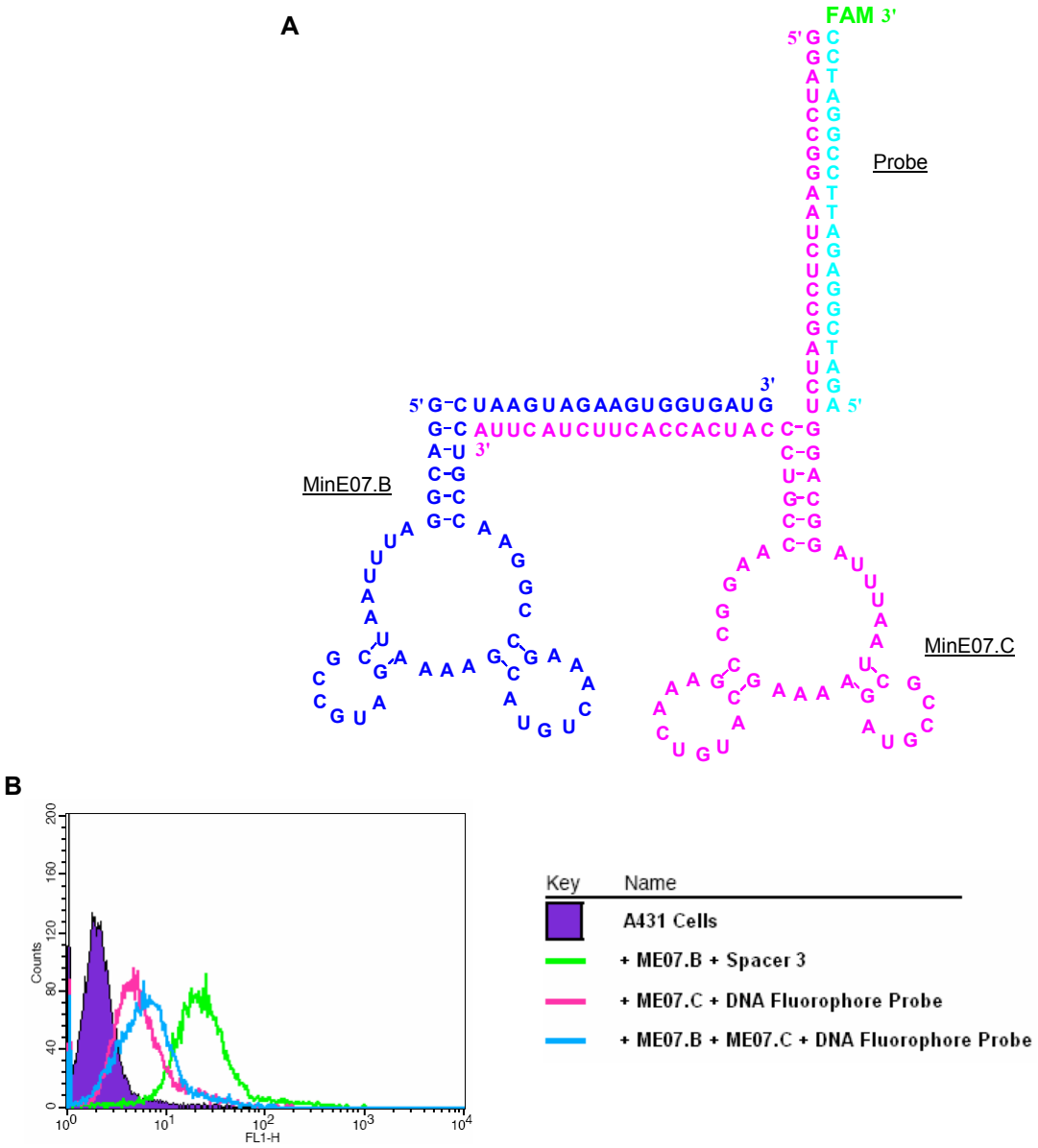

Figure 4: Fluorescein-bound aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.B annealed to DNA organizer Spacer 3 (green line), MinE07.C annealed to Probe (pink line), and MinE07.B and MinE07.C, and Probe annealed together (blue line). (**A**): Blue sequence represents MinE07.B; pink sequence indicates MinE07.C; and turquoise sequence denotes Probe. (**B**): FACS data of Construct 2.

Flow Cytometry Analysis: Construct 3

Construct 3 was formed by annealing MinE07.A and MinE07.B to SMCC DNA organizer. SMCC has been used as a common heterobifunctional linker with various compounds.[24] Similar to

Construct 1 except in the length and composition of the spacer between the hybdridization arms of the DNA organizer and orientation, Construct 3 provided the best comparison of orientation effects on binding affinity. FACS data (**Figure 5**) indicated no significant avidity effects from the dimer construct. However, a greater rightward shift in fluorescence was observed from MinE07.A bound to SMCC DNA organizer than when MinE07.A was annealed to any of the Spacers.

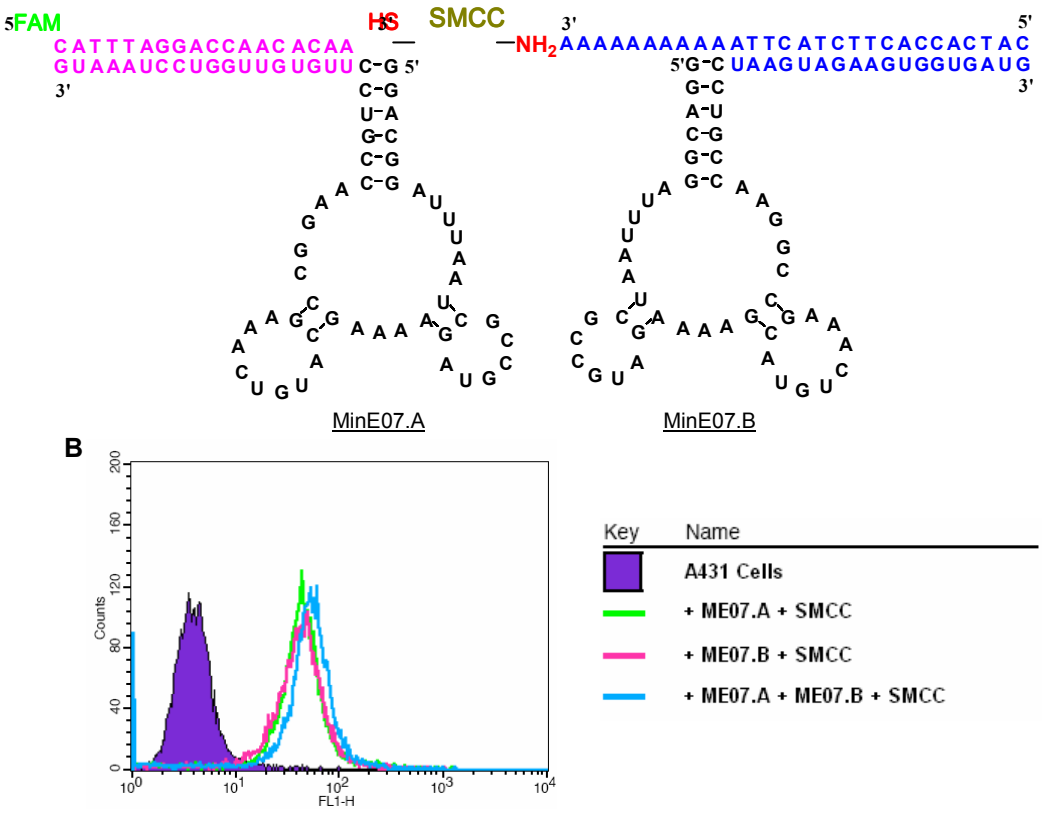

Figure 5: Fluorescein-bound aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.A annealed to SMCC DNA organizer (green line), MinE07.B annealed to SMCC DNA organizer (pink line), and MinE07.B and MinE07.C annealed to SMCC DNA organizer (blue line). (**A**): Pink sequences represent the hybridization between MinE07.A and its complement in SMCC DNA organizer; blue sequences indicate the hybridization between MinE07.B and its complement in SMCC DNA organizer. (**B**): FACS data of Construct 3.

As stated above, no significant avidity effects were seen with the dimerized aptamer construct. Other FACS data (not shown) did seemingly show slight avidity effects. However, inability to consistently observe these results led to the conclusion that Construct 3 does not show

significant avidity effects. Later experiments (data not shown) reveal that the state of the cells (confluent or in growing stage) and whether FACS is performed with attached cells (bound to culturing flasks or wells) or unattached cells (trypsinized cells) make a huge impact upon binding affinity of aptamers. The binding affinity of MinE07 is greatly reduced with unattached cells and with cells that are either overgrown or confluent (data not shown).

Flow Cytometry Analysis: Construct 4

Construct 4 was oriented head-to-tail with a variable intra-aptamer distance to observe whether spacing between aptamers had an effect on binding of dimeric constructs to EGFR. Construct 1 had indicated that binding affinity is not affected by intra-aptamer space. This conclusion was affirmed by FACS data from Construct 4 using Linker.B.5, B.10, and B.15 (**Figure 6**).

Annealing MinE07.B to Linker.A did not result in a shift in fluorescence, as expected, since Linker.A has no sequence that is complementary to MinE07.B. However, annealing MinE07.A to Linker.A, which share complementary sequences, also produced no shift in fluorescence, indicating the free arm of Linker.A might be interacting with something else. Annealing each individual aptamer to Linker.A and Linker.B resulted in a very slight shift, similar to that observed with MinE07.C and Probe. However, in this construct, the extensions made to MinE07 did not result in a three-dimernsional structure that reduced the binding affinity of the extended aptamer to EGFR since the same extensions that were used in forming the dimeric Constructs 1 and 3 were used to form Construct 4. Thus, the DNA organizers used to form Construct 4 must be responsible for the reduced affinity. No other complimentary regions than those indicated (**Figure 6A**) exist, and thus, there is a good possibility that the large size of the extended aptamers annealed to the Linkers (A and B) caused this decrease in binding affinity. Similar to Construct 3, the dimeric and monomeric forms resulted in approximately the same shift.

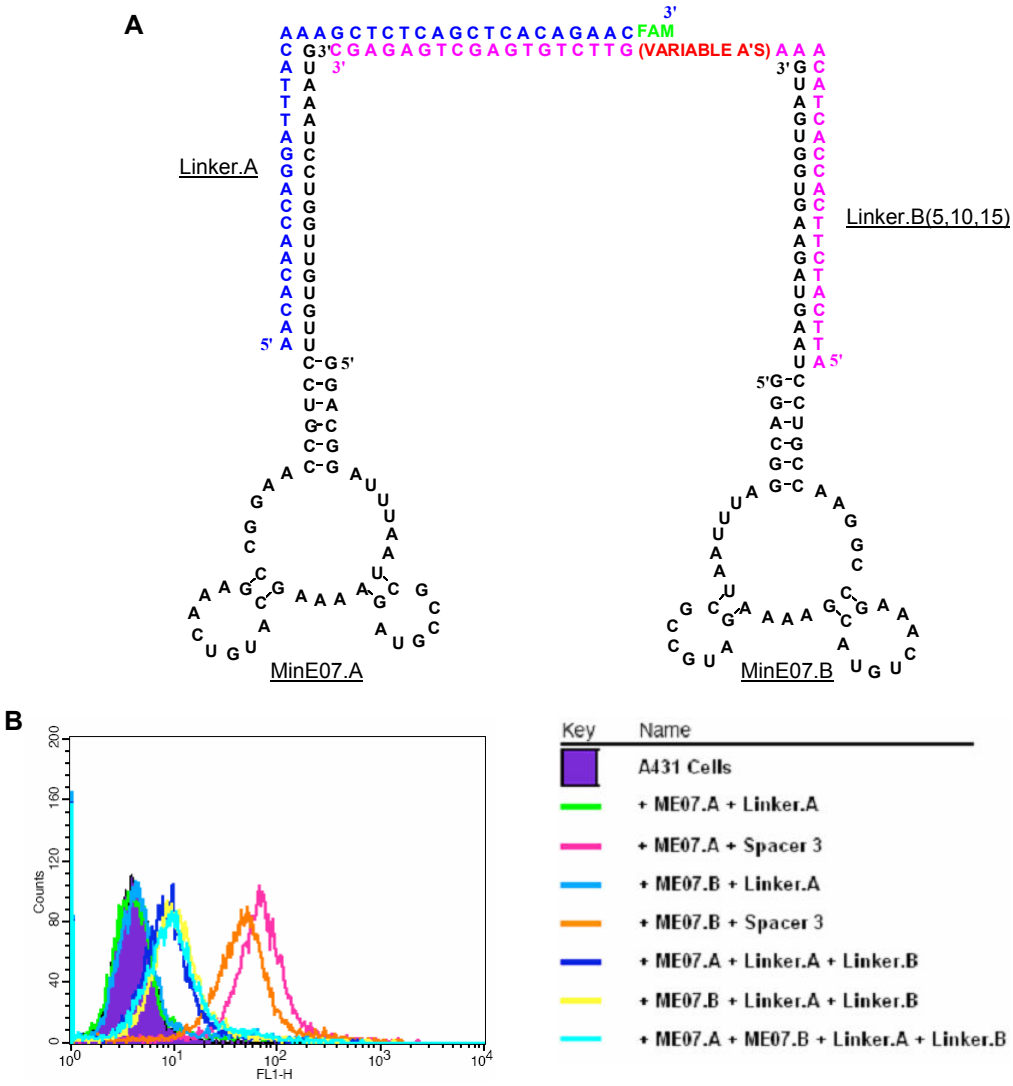

Figure 6: Fluorescein-bound aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.A annealed to Linker.A (green line), MinE07.A annealed to Spacer 3 (pink line), MinE07.B annealed to Linker.A (blue line), MinE07.B annealed to Spacer 3 (orange line), MinE07.A annealed to Linker.A and Linker.B (dark blue line), MinE07.B annealed to Linker.A and Linker.B (yellow line), and MinE07.A and MinE07.B annealed to Linker.A and Linker.B (light blue line). (**A**): Blue sequence represents Linker.A that hybridizes to MinE07.A; pink sequence indicates Linker.B that hybridizes to MinE07.B. (**B**): FACS data of Construct 4. Linker.B is a composite of all Linker.Bs (B.5, B.10, and B.15); all showed similar shift in fluorescence (data not shown).

Flow Cytometry Analysis: Construct 5

To remove any possible monomeric form of the aptamer in the population used to dose A431 cells, a ssDNA version of Construct 5 was ordered from IDT. After PCR was used to make the

second strand, it was transcribed into its modified RNA form. As mentioned earlier, Construct 5 contains both the full-length E07 and minimized E07 aptamers. Both have similar binding affinities (unpublished data). Given the way in which Construct 5 was made, it resulted in a head-to-head orientation (like Construct 1). Also, given that this dimeric construct did not have any extensions, no fluorescent molecule could bind to it. Thus, a competition assay was performed in which a fluorescent ligand of EGFR (MinE07.A annealed to Spacer 3) was competed off using MinE07 and Construct 5. FACS data (**Figure 7**) reveals that Construct 5 is much less effective in competing off MinE07.A annealed to Spacer 3 than MinE07 is. A four-fold higher concentration of Construct 5 and MinE07 than MinE07.A annealed to Spacer 3 was also used to observe a concentration-dependent shift.

The data implies that the head-to-head orientation is less effective than the head-to-tail orientation (as also indicated by the comparison between Constructs 1 and 3). The four-fold higher concentrations moved the fluoresnce shift leftward as expected, though it was still not sufficient to completely shift fluorescence back to baseline as established by A431 cells.

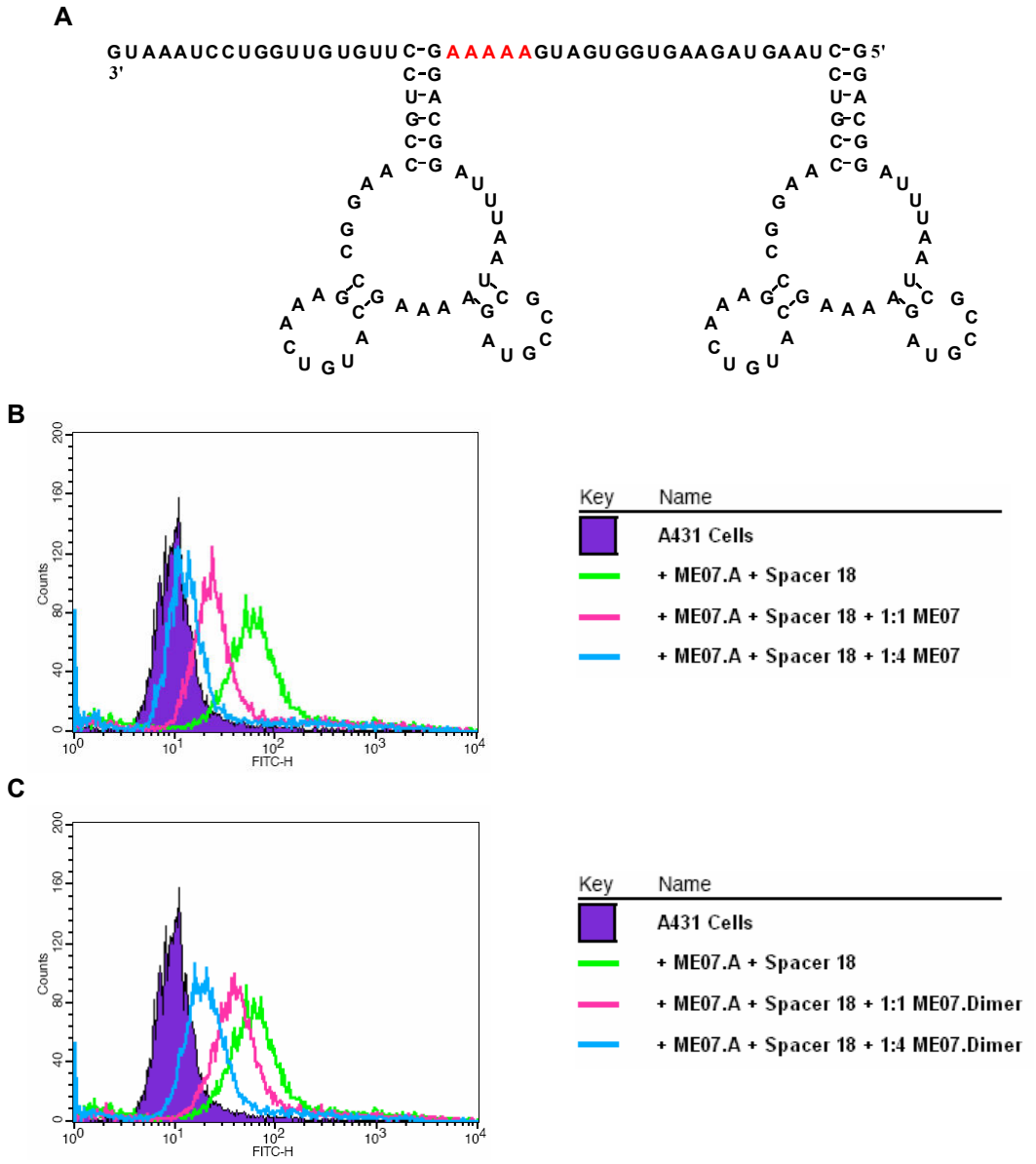

Figure 7: Competition assay using aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.A annealed to Spacer 18 (green line), MinE07 or MinE07.Dimer competing off MinE07.A annealed to Spacer 18 (pink line), and four-fold higher concentration of MinE07 or MinE07.Dimer competing off MinE07.A annealed to Spacer 18 (blue line). (**A**): Red sequence represents the 5 As separating MinE07 (to the left) and E07 (to the right. (**B**): FACS competition data using MinE07. (**C**): FACS competition data using MinE07.Dimer.

Flow Cytometry Analysis: Mutant Minimized Aptamers

To verify that the sequence of the extended aptamers is responsible for the shifts in fluorescence, mutant minimized aptamers with different sequences than the minimized aptamer

were extended using the same 3' extensions as those used for MinE07.A and MinE07.B (**Table 3**). The mutant constructs were assayed by annealing the mutant aptamers to Spacer 18 DNA organizer (same form as Construct 1). FACS data (**Figure 8**) proved that it was indeed the aptamer sequence that mediated binding to EGFR and the subsequent shift in fluorescence.

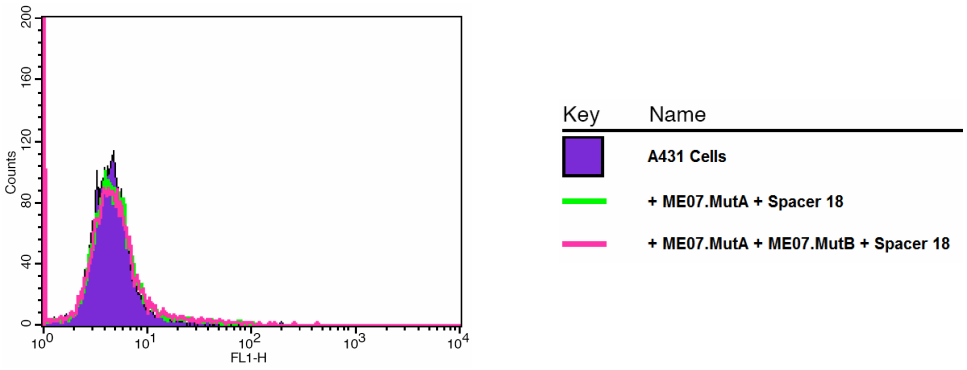

Figure 8: Fluorescein-bound mutant aptamer construct-mediated binding to A431 cells (ME07 refers to MinE07): MinE07.MutA annealed to Spacer 18 (green line) and MinE07.MutA and MinE07.MutB annealed to Spacer 18 (pink line). FACS data of mutant aptamers using Spacer 18 (similar formation as observed in Construct 1 (**Figure 2A**).

The mutant aptamer assay was essentially a proof-of-principle used to verify the results of the other FACS assays.

Comparison of Dimer Constructs

The dimer constructs used in this experiment were oriented either head-to-head (with 5' ends facing the same direction) or head-to-tail (with 5' ends facing in opposite directions): Constructs 1 and 5 were oriented head-to-head and Constructs 2, 3, and 4 were oriented head-to-tail. The head-to-tail orientation seems to have provided the best possibility for avidity effects (as suggested by Construct 3). Considering that EGFRs dimerize in a head-to-tail orientation, having aptamers in a head-to-tail orientation would facilitate EGFR dimerization.[25-26] Another variable that was studied was the spacing between the aptamers (by varying the distance between the hybridization arm sequences) to see if intra-aptamer distance affected binding

affinity to two EGFRs. Constructs 1 and 4 indicate that the intra-aptamer distance does not significantly affect binding affinity. Nonetheless, no definite conclusions can be drawn about the spacing of EGFRs on the cell surface from this experiment. Since no avidity effects were clearly observed, dimerization of EGFR may not have occurred and thus, intra-aptamer distance may not have made a significant difference. Complicating the matter, however, is the fact that more than 40% of EGFRs exist as dimers prior to stimulation by ligand.[26] Pull-down experiments or studying downstream protein activity may reveal whether EGFR dimerization was induced by dimer constructs or not.[27-29]

It may also have been interesting to study the affect of dimerization on internalization efficiency of anti-EGFR aptamers.[30] Approximately 15% of E07 that is bound to EGFR is also internalized (unpublished data). It is possible that more or less of the Constructs were internalized due to dimerization. A nuclease digestion treatement (as with Riboshredder) would have indicated internalization efficiency.[17,31] Cell microscopy would have also revealed whether dimeric constructs internalized better than monomeric constructs.[32-34]

Although no avidity was seen with any of the constructs used, Construct 3 did provide some evidence that avidity is possible under certain conditions. Increasing the distance between the aptamers (by increasing the length of an internal spacer or the distance between the hybridization arm sequences) may affect binding affinity once avidity can be consistently reproduced. Also, the EGFR pathway in A431 cells (which overexpress EGFR) is known to be different than in cells expressing EGFR at normal levels.[33] However, given that cancerous cells will generally overexpress EGFR, A431 cells may be the better model.

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REFERENCES

1. Egloff A.M., Grandis, J.R. Targeting EGFR and Src pathways in head and neck cancer. (2008) Seminar Oncol. **35**(3): 286-297.

2. Flynn J.F., Wong C., Wu, J.M. (2009) Anti-EGFR therapy: Mechanism and advances in clinical efficacy in breast cancer. J. Oncol. **2009**: 526963.

3. Karapetis C.S., Khambata-Ford S., Jonker D.J., O'Callaghan C.J., Tu D., Tebbutt N.C., Simes R.J., Chalchal J., Shapiro J.D., Robitaille S., Price T.J., Shepherd L., Au H.J., Langer C., Moore M.J., Zalcberg J.R. *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. (2008) NJEM. **359**(17): 1757-1765.

4. Ladanyi M., Pao W. (2008) Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. Modern Pathology. **21**: S16-S22.

5. Yang S.X., Simon R.M., Tan A.R., Nguyen D., Swain S.M. (2005) Gene expression patterns and profile changes pre- and post- Erlotinib treatment in patients with metastatic breast cancer. Clin. Cancer. Res. **11**(17): 6226-6232.

6. Göstring L., Chew M.T., Orlova A., Höidén-Guthenberg I., Wennborg A., Carlsson J., Frejd F.Y. (2009) Quantification of internalization of EGFR-binding Affibody molecules: Methodological aspects. International J. Oncol. **36**: 757-763.

7. Nimjee S.M., Rusconi C.P., Sullenger B.A. (2005) Aptamers: An emerging class of therapeutics. Annuv. Rev. Med. **56**: 555-83.

8. Arora A., Scholar E.M. Role of tyrosine kinase inhibitors in cancer therapy. (2005) JPET. **315**: 971-979.

9. Que-Gewirth, N.S., Sullenger B.A. (2007) Gene therapy progress and prospects: RNA aptamers. Gene Therapy. **14**: 283-291.

10. Hermann T., Patel D.J. (2000) Adaptive recognition by nucleic acid aptamers. Science **287**: 820-825.

11. Blind M., Blank M. (2005) Aptamers as tools for target validation. Current Opinion in Chemical Biology. **9**: 336-342.

12. Famulok M., Hartig J.S., Mayer G. (2007) Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. Chemical Reviews **107**: 3715-3737.

13. Chu T.C., Twu K.Y., Ellington, A.D., Levy,M. (2006) Aptamer mediated siRNA delivery. Nucleic Acids Res. **34**(10), e73.

14. Li N., Ebright J.N., Stovall G.M., Chen X., Nguyen H.H., Sing A., Syrett A., Ellington A.D. (2008) Technical and biological issues relevant to cell typing with aptamers. J. Proteome Res. **8**: 2438-2448.

15. Boese B.J., Breaker B.R. (2007) *In vitro* selection and characterization of cellulose-binding DNA aptamers. Nucleic Acids Res. **35**(19): 6378-6388.

16. Crow M.J., Grant G., Provenzale J.M., Wax A. (2009) Molecular imaging and quantitative measurement of epidermal growth factor receptor expression in live cancer cells using immunolabeled gold nanoparticles. AJR Am. R. Roentgenol. **192**(4): 1021-8.

17. Li N., Larson, T., Nguyen H.H., Sokolov K.V., Ellington A.D. (2010) Directed evolution of gold nanoparticle delivery to cells. Chem. Commun. **46**: 392-394.

18. Wolff E.A., Schreiber G.J., Cosand W.L., Raff H.V. (1993) Monoclonal antibody homodimers: Enhanced antitumor activity in nude mice. Canc. Res. **53**: 2560-2565.

19. Charles C.H., Luo G.X., Kohlstaedt L.A., Morantte I.G., Gorfain E., Cao L., Williams J.H., Fang F. (2003) Prevention of human rhinovirus infection by multivalent Fab molecules directed against ICAM-1. Antimicrobial Agents and Chemotherapy. **47**(5): 1503-1508.

20. Kubetzko S., Ballc E., Walbel R., Zangemeister-Wittke W., Plückthun A. (2006) PEGlyation and multimerization of the anti-p185^{HER-2} single chain Fv fragment 4D5. Effects on Tumor Targeting. J. Bio. Chem. **281**(46): 35186-35201.

21. Shiroshi M., Kuroki K., Ose T., Rasubala L., Shiratori I., Arase H., Tsumoto K., Kumagai I., Kohda D., Maenaka K. (2006) Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer. J. Bio. Chem. **281**(15): 10439-10447.

22. Zuker M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**(13): 3406-15.

23. Cowperthwaite M.C., Ellington A.D. (2008) Bioinformatic analysis of the contribution of primer sequences to aptamer structures. J. Mol. Evol. **67**(1): 95-102.

24. Li J., Lee S.E., Belciug M., Ring D.B., Kwok C.S. (2000) Chemical conjugation of a novel antibody-interleukin 2 immunoconjugate against c-erb-2 product. Chin. Med J. (Engl) **113**(2): 151-3.

25. Kästner J., Loeffler H.H., Roberts S.K., Martin-Fernandez M.L., Winn M.D. (2009) Ectodomain orientation, conformational plasticity and oligomerization of ErbB1 receptors investigated by molecular dynamics. J. Struct. Biol. **167**(2): 117-28.

26. Bader A.N., Hofman E.G., Voortman J., en Henegouwen P.M., Gerritsen H.C. (2009) Homo-FRET imaging enables quantification of protein cluster sizes with subcellular resolution. Biophys. J. **97**(9): 2613-22.

27. Liu W.J., Chang Y.S., Wang H.C., Leu J.H., Kou G.H, Lo C.F. (2008) Transactviation, dimerization, and DNA-binding activity of white spot syndrome virus immediate-early protein IE1. J. Virol. **82**(22): 11362-11373.

28. Schulte R., Talamas J., Doucet C., Hetzer M.W. (2008) Single bead affinity detection (SINBAD) for the Analysis of protein-protein interactions. PLoS ONE. **3**(4): e2061.

29. Li H., Yuan M., Mao T. (2007) AtMAP65-1 binds to tubulin dimers to promote tubulin assembly. J. Biochem. Mol. Bio. **40**(2): 218-225.

30. Chu T., Ebright J., Ellington A. D. (2007) Using aptamers to identify and enter cells. Curr. Opin. Mol. Ther. **9**(2): 137-44.

31. Xiao Z., Shangguan D., Cao Z., Fang X., Tan W. (2008) Cell-Specific Internalization Study of an aptamer from whole cell selection. Chem. Eur. J. **14**: 1769-1775.

32. Shangguan D., Li Y., Tang Z., Cao Z.C., Chen H.W., Mallikaratchy P., Sefah K., Yang C.J., Tan W. (2006) Aptamers evolved from live cells as effective molecular probes for cancer study. Proc. Natl. Acad. Sci. U.S.A **103**(32): 11838-43.

33. Bryant J.A., Finn R.S., Slamon D.J., Cloughesy T.F., Charles A.C. (2004) EGF activates intracellular and intercellular calcium signaling by distinct pathways in tumor cells. Cancer Biol. Ther. **3**(12): 1243-9.

34. Jiang H., Zhang J., Shi B.Z., Xu Y.H., Li Z.H., Gu J.R. (2007) Application of EGFP-EGF fusions to explore mechanism of endocytosis of epidermal growth factor. Acta. Pharmacol. Sin. **28**(1): 111-7.